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Increased Hepatic Lipid Soluble Antioxidant Capacity as Compared to Other Organs of Streptozotocin-Induced Diabetic Rats: A Cyclic Voltammetry Study

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It has been suggested that oxidative stress plays an important role in the chronic complications of diabetes. The experimental findings regarding the changes in tissue antioxidant enzymes and lipid peroxidation of diabetic tissues have been inconsistent. Previous studies in our laboratory demonstrated that the reducing power of a specific tissue correlates with its low molecular weight antioxidant (LMWA) capacity. In the present study, the overall LMWA capacity (reducing equivalents) of plasma and tissues of streptozotocin (STZ)-induced diabetic rats (1–4 weeks) and insulin treated diabetic rats were measured by cyclic voltammetry. Levels of water and lipid soluble LMWA capacity progressively decreased in the diabetic plasma, kidney, heart and brain, while the diabetic liver, at 2, 3 and 4 weeks after STZ injection, showed a significant increase in the overall lipid soluble LMWA capacity ($p < 0.001$). Subsequently, analysis of specific components by high pressure liquid chromatography (electrochemical detection) showed decreased levels of ascorbic acid in plasma, kidney, heart and brain of diabetic animals. The α-tocopherol level dropped in all tissues, except for the liver in which there was a significant increase ($p < 0.01$ and $p < 0.001$ at 2–4 weeks). Lipid peroxidation was assessed by conjugated diene levels, which increased significantly in all diabetic tissues except the liver. Insulin treatment that was started after 3 weeks of diabetes and continued for 3 weeks showed no change in the conjugated dienes and in the overall LMWA capacity in all organs. Our results suggest a unique behavior of the liver in the STZ-induced diabetic rats to the stress and indicate its higher capacity to cope with oxidative stress as compared to other organs.

Keywords: Low molecular weight antioxidant capacity, cyclic voltammetry, oxidative stress, reducing equivalents, vitamin C, α-tocopherol, liver

INTRODUCTION

Oxidative stress occurs when there is an imbalance between free radical reactions and the scavenging capacity of antioxidative defense mechanism of the organism.[1] Patients with diabetes

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may be especially more prone to oxidative stress which enhances the development of diabetic complications.[2,3] It is suggested that persistent hyperglycemia in diabetes enhances the production of reactive oxygen species (ROS) from glucose autooxidation[4] and protein glycation,[5] which leads to tissue damage.

The tissue level of antioxidants critically influences the susceptibility of various tissues to oxidative stress and is associated with the development of complications of diabetes.[2,3] However, the role of oxidative stress in the initiation and progression of diabetes remains uncertain. Changes in the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione-related enzymes (GST and GR) were previously shown to assess the oxidative stress in the diabetic tissues. However, there is no agreement on these enzyme alterations.[6–9] Similarly, there are conflicting reports on changes in specific LWMA such as α-tocopherol (Vitamin E), ascorbic acid and glutathione.[10–13] Greater resistance to lipid peroxidation was observed in diabetic liver, kidney and heart.[14,15]

Since the total antioxidant capacity of plasma or tissue is derived from the known and unknown antioxidants, we have suggested that measurement of the overall antioxidant profiles, rather than specific compounds, is the appropriate approach for investigating hyperglycemia-induced oxidative stress. Therefore, in the present study we attempted to evaluate the total scavenging activity of rat organs and in particular of the liver in streptozotocin (STZ)-induced diabetic rats. This was achieved by measuring the overall reducing power, which correlates with the overall scavenging activity, by cyclic voltammeter (CV) methodology.[16–20] The CV tracings record the peak potential (specific to the type of scavenger(s) present) and the anodic current (*Ia*), which depends on the scavenger concentration(s).

The result of the present study showed that diabetic liver displayed an increase in the overall lipid soluble antioxidant capacity and a parallel resistance towards lipid peroxidation. The other organs of the diabetic tissues showed significantly decreased levels.

MATERIALS AND METHODS

Induction of Diabetes

Male Sabra rats (Wistar outbred rats, Hebrew University strain) weighing 200–230 g were used in this study. Animals were maintained according to the regulations of the Animal Care Committee of The Hebrew University, with 12-h light/dark reversed light cycle, and food and water provided *ad libitum*. Diabetes was induced in overnight fasted rats using STZ (Sigma, St. Louis, USA).[21] STZ was dissolved in citrate buffer (0.1 M, pH 4.5) and administered intraperitoneally (i.p.) at a dose of 65 mg/kg. The STZ-injected rats were given a 5% glucose solution to overcome drug-induced hypoglycemia. Diabetes was verified 72 h later by estimating hyperglycemia and glucosuria (Diastix). Blood for glucose determination during the experiment, was taken from the tail vein, using the glucometer (MEDISENSE glucometer, UK). Control rats received an equivalent amount of buffer i.p. Animals with blood glucose levels higher than 15 mmol/l and with little or no weight gain were included in the study. Animals not meeting these criteria, e.g. hyperglycemia but showing weight gain, were omitted from the study. Body weight and blood glucose were recorded weekly.

Experiment 1 Control and diabetic rats were sacrificed at weekly intervals for 4 weeks to study the changes in the total LMWA capacity during the progression of diabetes (six rats per group per week).

Experiment 2 A group of ($n = 20$) STZ-injected diabetic animals were used for the insulin replacement study. Diabetic animals were kept untreated for 3 weeks. At the end of third week diabetic animals were divided into two groups. One group of diabetic animals received a single daily injection (s.c) of 9–12 U/kg of human ultratard insulin, with additional fast acting

insulin (Actrapaid) if required. The dosage of insulin was adjusted by monitoring urinary glucose daily and blood glucose weekly to prevent glucosuria and hyperglycemia. The insulin treatment was continued for 3 weeks. Untreated diabetic and insulin treated diabetic animals and normal control animals ($n = 6$/each group) were sacrificed at the end of sixth week.

Animals were killed by cervical decapitation, and blood was collected using heparin as an anticoagulant. Tissues (liver, heart, kidney and brain) were rapidly excised, weighed and washed in ice cold saline and stored at $-70°C$ until further use.

Sample Preparation for Cyclic Voltammetry Analysis

To determine the overall reducing power of the various tissues, 500 mg samples of tissue (liver, kidney, heart and brain) were homogenized in 5 ml of 0.1 M phosphate buffered-saline (PBS), pH 7.2. Following homogenization, the samples were centrifuged at 1000*g* for 10 min at 4°C to remove large, insoluble particles. Aliquots were removed for protein determination and the remaining samples were divided into two parts (1 and 4 ml). The smaller part (1 ml) was used to measure water soluble LMWA. Lipophilic LMWA were extracted from the second sample. In brief, the 4 ml tissue samples were homogenized in methanol : hexane (1 : 4) and recentrifuged at 1000*g* for 10 min. The upper and lower layers were separated and the organic solvents were removed by evaporation. The residue was dissolved in acetonitrile : methanol (1 : 1) containing 1% tetrabutyl ammonium perchlorate (ion-pairing agent), and the samples were then subjected to cyclic voltammetry.

Measurement of Plasma and Tissue Reducing Capacity

A BAS model CV-1B cyclic voltammeter (West Lafayette, IN, USA) was used to evaluate the reducing power of the plasma and tissue samples.[20] CV tracings were recorded at a range of 0–1.3 V and at a rate of 100 mV/s vs. an Ag/AgCl reference electrode. A three electrode system was used throughout the study. The working electrode was a glassy carbon disk (BAS MF-2012) of 3.2 mm diameter. A platinum wire served as the counter electrode. The working electrode was polished prior to each measurement with a polishing kit (BAS-PK-1). CV tracings were analyzed to determine peak potential ($E_{1/2}$) and anodic current (*Ia*). The peak potential was measured at the half increase of the anodic wave (see Figure 1). This potential is typical of the tested tissue and represents the ability of the tissue's reducing equivalents, composing the anodic wave, to donate electrons to the working electrode. The anodic current (*Ia*) correlated with the concentration of the reducing equivalents. The buffer (PBS) itself did not show any anodic wave up to 1.3 V. At least three tracings were recorded for each sample, and the repeated scans yielded similar CV tracings. A change of >50 mV in peak potential was considered significant[20] and was attributed to the presence of another reducing equivalent or group of LMWA, possessing a different peak potential.

HPLC-ECD Measurements

To evaluate the various compounds composing the CV waves, tissue samples were treated with 25% TCA to precipitate out proteins, and 20 µl of deproteinized samples were injected into an HPLC system (Kontron, Switzerland). The column was connected to a LC4A amperometric electrochemical detector (BAS, West Lafayette, IN, USA) with a glassy carbon working electrode and an Ag/AgCl reference electrode. A mobile phase consisting of 40 mM sodium acetate buffer, pH 4.75, 0.54 mM Na_2EDTA and 1.5 mM tetrabutyl ammonium hydroxide was used for analysis of water soluble antioxidants. A mobile phase containing 20 mM lithium perchlorate in methanol : ethanol : isopropanol (22.5 : 73.6 : 3.9) was

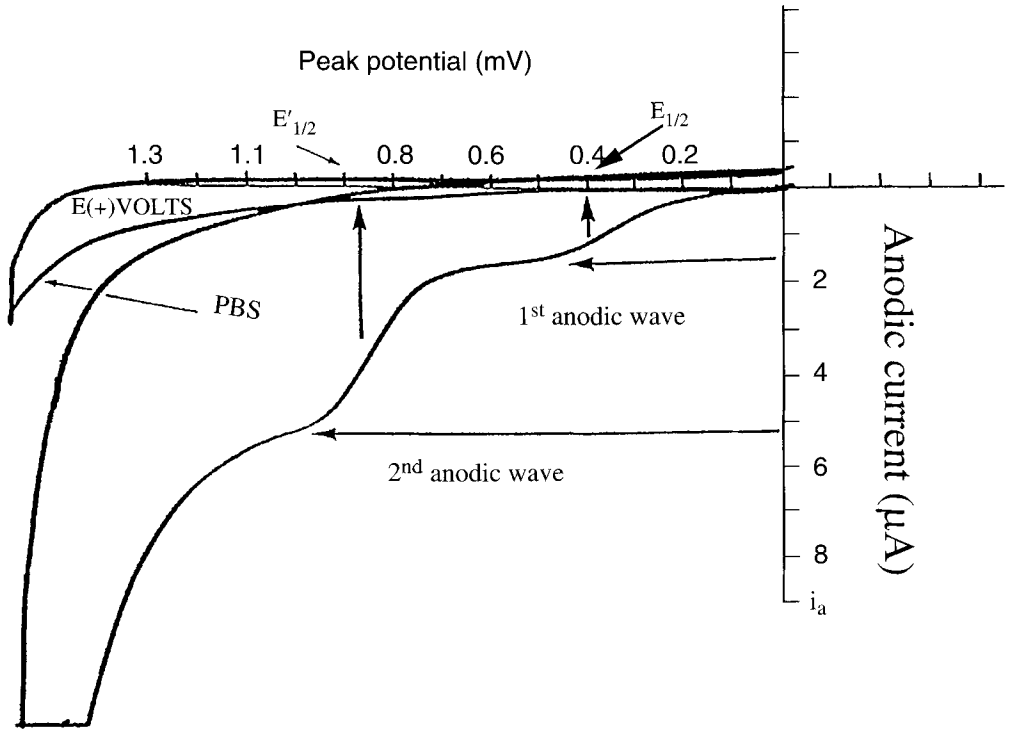

FIGURE 1 A representative cyclic voltammogram of control liver homogenate in PBS. Two anodic waves were recorded at peak potentials 400 and 890 mV (denoted by $E_{1/2}$ and $E'_{1/2}$). The total concentration of the reducing equivalents is determined from the y axis (anodic current (Ia)). A voltammogram of PBS solution is shown, with no recorded peak potentials. CV tracings were performed as described in the Materials and Methods.

used to analyze the lipid soluble antioxidants.[22] Separations were performed at a flow rate of 1 ml/min and the chromatograms were recorded on a PC-based data acquisition and processing system. Standards (ascorbic acid, uric acid, α-tocopherol and β-carotene) were injected before the samples.

Measurement of Conjugated Diene

Oxidative damage was evaluated by measuring the conjugated diene levels and it was assessed by measuring optical density (OD) in the ultraviolet range according to Buege and Aust[23] with minor modifications. In brief, tissue samples were homogenized in 4% NaCl (10% w/v) and extracted with eight volumes of a mixture of $CHCl_3$ and methanol (2 : 1). Centrifugation for 10 min at 1900 *g* allowed separation of the lipid fraction into the lower chloroform phase. Aliquots of lipid chloroform extract were evaporated at 45°C under a stream of dry nitrogen. The total lipid extracts were redissolved in 1 ml of spectrograde cyclohexane. The spectrum was then scanned between 220 and 330 nm, and the amount of conjugated diene was calculated using an extinction coefficient of $2.52 \times 10^4\,M^{-1}\,cm^{-1}$. Conjugated dienes were expressed as μmoles per gram of tissue.

Statistics

Values of peak potential and anodic current are expressed as the mean ± SD of at least six animals per group. One-way analysis of variance was performed to compare the different anodic

currents at the various time points within a particular group. The Student '*t*' test was used for comparison among the different groups. The level of significance was $p < 0.05$ (Instat, Graph pad, San Deigo, CA, USA).

RESULTS

Blood Glucose and Body Weight Changes

Blood glucose levels were estimated 3 days after STZ injection. Almost 85% of the STZ-injected animals showed hyperglycemia, with blood glucose levels higher than in the control animals (control 5.6 ± 0.63 mmol/l, diabetic 18 ± 3.2 mmol/l). Blood glucose remained unchanged in the control group but increased progressively in the diabetic group, reaching 25–30 mM/l after 2–3 weeks. Blood glucose was significantly higher ($p < 0.001$) in the diabetic group compared with the control group at all times.

Control and insulin treated diabetic animals showed a progressive and significant increase in the body weight whereas untreated diabetic animals showed a significant decrease ($p < 0.001$) in the body weight compared with their controls at all times. In the sixth week control, untreated diabetic and insulin treated diabetic animals showed a body weight of 290 ± 15, 220 ± 5, 280 ± 15 g, respectively, $p < 0.001$ control vs. diabetic; diabetic vs. insulin treated diabetic.

CV Systems and its Components

A representative CV obtained from control liver (PBS homogenate) is presented in Figure 1. There are two anodic waves, the first appearing at 400 ± 20 mV and the second at 890 ± 25 mV. These waves reflect the oxidation potential ($E_{1/2}$) of the tissue. Wave current height (*Ia*) is proportional to the concentration of component(s) being oxidized at the specific potential. At 400 mV, control liver had an anodic current 0.34 ± 0.023 μA/mg protein, and at the second anodic potential (890 ± 25 mV), anodic current was (*Ia*) $= 0.64 \pm 0.025$ μA/mg protein.

CV Analysis of Water Soluble LMWA of Rat Organs Following Induction of Diabetes

The plasma and PBS homogenates of most of the tissues showed two anodic waves, whereas the heart displayed only one anodic wave. When the CV tracings were recorded at different time points after induction of diabetes, the peak potentials ($E_{1/2}$) did not change indicating that the types of LMWA composing the waves were not altered considerably. However, significant changes in the levels of anodic current (*Ia*) were observed in the diabetic animals, indicating changes in total LMWA concentrations. Table I shows the anodic current changes in liver (PBS homogenate) of control and diabetic rats (1–4 weeks). A significant decrease in the anodic current was

TABLE I Water soluble LMWA antioxidant capacity of rat liver: Anodic current changes in control and diabetic rat liver (1–4 weeks). Anodic current was calculated from the cyclic voltammetric tracings as described in the Materials and Method. Values are expressed as mean ± SD for six animals in each group

Week of Diabetes (Liver)	First potential (mV)	First anodic current (μA/mg protein)		Second potential (mV)	Second anodic current (μA/mg protein)	
		Control	*Diabetic*		*Control*	*Diabetic*
1st	(400 ± 20)	0.34 ± 0.025	0.33 ± 0.022	(890 ± 25)	0.64 ± 0.025	0.63 ± 0.014
2nd		0.35 ± 0.023	0.25 ± 0.018***		0.65 ± 0.023	0.62 ± 0.017
3rd		0.35 ± 0.022	BDL		0.65 ± 0.025	0.45 ± 0.013***
4th		0.36 ± 0.025	BDL		0.65 ± 0.024	0.42 ± 0.015***

***$p < 0.001$, control vs. diabetic; BDL – below detection limit.

observed in the diabetic liver between the second and fourth weeks, with disappearance of the first anodic wave in the third and fourth weeks, indicating a dramatic drop in the concentration of water soluble reducing equivalents (scavengers).

Control plasma displayed two anodic waves at 400 ± 15 and 920 ± 25 mV, with anodic currents of $(Ia) = 0.24 \pm 0.012$ and 0.44 ± 0.026 μA/mg protein, respectively. In diabetic plasma, a significant, progressive decrease in anodic current at both potentials was observed, starting with the second week. A significant decrease in the first $(Ia) = 0.11 \pm 0.01$ μA/mg protein ($p < 0.001$) and second anodic current $(Ia) = 0.28 \pm 0.01$ μA/mg protein ($p < 0.001$), respectively was noted in the fourth week of diabetic plasma. The control kidney showed two anodic waves at 400 ± 25 and 900 ± 25 mV, with anodic currents of $(Ia) = 0.2 \pm 0.012$ and 0.44 ± 0.022 μA/mg protein, respectively. In diabetic kidney, a significant progressive decrease in the anodic current at both potentials was observed, starting with the second week. Complete disappearance of first anodic wave and a significant decrease in the second anodic wave $(Ia) = 0.30 \pm 0.01$ μA/mg protein ($p < 0.001$) was noted in the fourth week diabetic kidney. There was no significant change in the anodic current in the brain and heart tissues of second and third week diabetic animals, whereas a significant decrease ($p < 0.05$) was observed in these organs 4 weeks after induction of diabetes. Control and fourth week diabetic heart (800 ± 25 mV) showed an anodic current $(Ia) = 0.54 \pm 0.02$ and 0.50 ± 0.02 μA/mg protein ($p < 0.05$).

CV Analysis of Lipophilic LMWA of Rat Organs Following Induction of Diabetes

Similar CV tracings were recorded in lipid extracts of both control and diabetic tissues. Lipid extracts of the samples revealed two anodic waves, indicating two groups of lipid soluble LMWA (Table II). Lipids extracted from the control liver displayed two anodic waves, at 315 ± 10 and 915 ± 12 mV ($(Ia) = 0.14 \pm 0.013$ and 0.24 ± 0.02 μA/mg protein, respectively). A significant increase in the second anodic current was observed in the diabetic liver starting from the second week (second week: $(Ia) = 0.44 \pm 0.02$μA/mg protein, third week: $(Ia) = 0.52 \pm 0.01$μA/mg protein, fourth week: $(Ia) = 0.83 \pm 0.02$ μA/mg protein, $p < 0.001$ vs. respective controls). No significant change in the first anodic wave was observed in diabetic liver. There was a significant decrease in lipid soluble LMWA in diabetic plasma, kidney, heart and brain.

Insulin Replacement on Water Soluble and Lipid Soluble LMWA

Figures 2 and 3 show the anodic current changes in the liver of control, diabetic (6 weeks) and insulin treated diabetic animals. Insulin treatment normalized the altered levels of LMWA.

TABLE II Lipid soluble LMWA antioxidant capacity of rat liver: Anodic current changes in organic extracts of control and diabetic rat liver (1–4 weeks). Anodic current was calculated from the cyclic voltammetric tracings as described in the Materials and Method. Values are expressed as mean ± SD for six animals in each group

Week of Diabetes (Liver)	First potential (mV)	First anodic current (μA/mg protein)		Second potential (mV)	Second anodic current (μA/mg protein)	
		Control	*Diabetic*		*Control*	*Diabetic*
1st	(320 ± 20)	0.14 ± 0.013	0.13 ± 0.012	(915 ± 25)	0.24 ± 0.015	0.23 ± 0.014
2nd		0.15 ± 0.012	0.15 ± 0.014		0.25 ± 0.013	0.44 ± 0.022***
3rd		0.15 ± 0.014	0.16 ± 0.013		0.25 ± 0.015	0.52 ± 0.014***
4th		0.16 ± 0.012	0.17 ± 0.023		0.25 ± 0.014	0.83 ± 0.017***

***$p < 0.001$, control vs. diabetic.

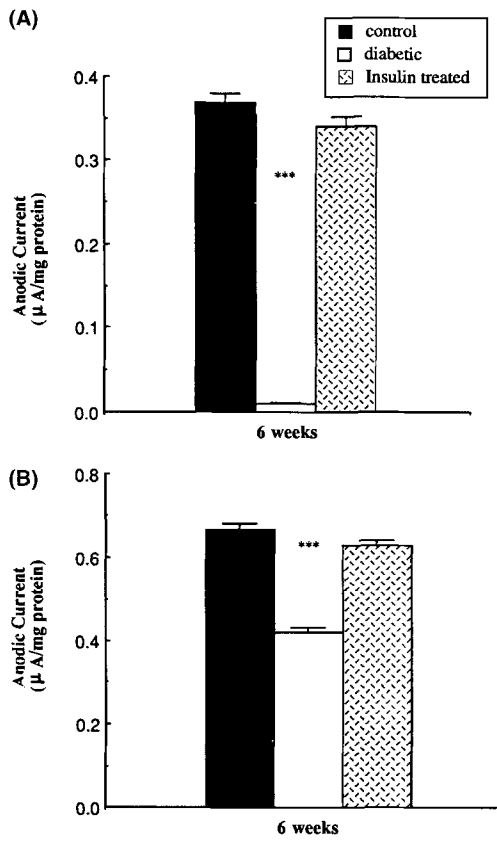

FIGURE 2 Anodic current (*Ia*) of first (400 ± 20 mV) (A) and second (920 ± 25 mV) (B) anodic wave of control, diabetic (6 week) and insulin treated diabetic liver (PBS homogenate). CV tracings were recorded as described in Materials and Methods. *Ia* was calculated as in Figure 1 and normalized per mg protein. $^{***}p < 0.001$ vs. the control; insulin treated.

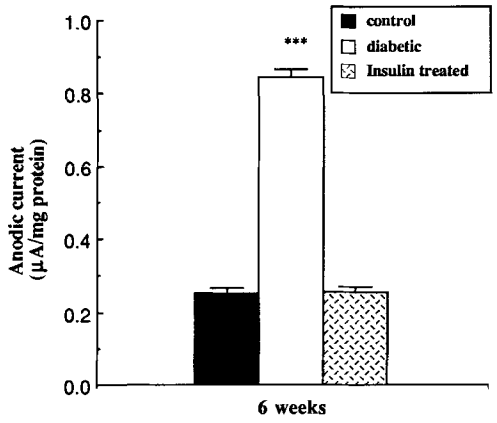

FIGURE 3 Anodic current (*Ia*) of first (320 ± 20 mV) (A) and second (915 ± 25 mV) (B) anodic wave of control, diabetic (6 week) and insulin treated diabetic liver (lipid extract). CV tracings were recorded as described in Materials and Methods. *Ia* was calculated as in Figure 1 and normalized per mg protein. $^{***}p < 0.001$ vs. the control; insulin treated.

TABLE III Ascorbic acid and α-tocopherol levels in control and diabetic liver (1–4 weeks). The values are expressed as the mean ± SD of six animals in each group

Week (Liver)	Vitamin C (μM)		Vitamin E (μM)	
	Control	*Diabetic*	*Control*	*Diabetic*
1st	125 ± 24	112 ± 20	7.3 ± 1.2	7.6 ± 1.3
2nd	125 ± 25	81 ± 20***	7.3 ± 1.4	9.4 ± 1.3**
3rd	130 ± 22	78 ± 19***	7.5 ± 1.4	10.2 ± 1.5**
4th	135 ± 26	67 ± 15***	7.6 ± 1.6	12.0 ± 1.8***

$^{**}p < 0.01$, $^{***}p < 0.001$, control vs. diabetic.

Similarly, insulin treated diabetic kidney, brain and heart showed no significant change in the anodic currents at both potentials when compared with their controls. Insulin treatment for 3 weeks normalized the altered levels of water and lipid soluble LMWA in all organs.

HPLC-ECD Analysis

HPLC-ECD analysis of specific water and lipid soluble antioxidants in control and diabetic liver is shown in Table III. A significant increase in the α-tocopherol level was observed in the second, third and fourth weeks of the diabetic liver ($p < 0.01$ and $p < 0.001$, respectively) while the liver ascorbic acid level was found to decrease suggesting that the increase in the second anodic wave (lipid extract) of the CV was due to an increase in α-tocopherol levels. A significant decrease in the ascorbic acid and α-tocopherol level was observed in plasma, kidney, heart and brain, whereas there was no significant change in the uric acid concentration. In the fourth week of diabetes, a decrease in ascorbic acid level of about 50% was observed in the plasma, liver and kidney whereas only 13% and 41% decrease was noted in the brain and heart, respectively. α-Tocopherol was found to decrease by 50% in

TABLE IV Conjugated diene levels in plasma and various tissues of control and diabetic (6 weeks) and insulin treated diabetic rat. The values are expressed as mean ± SD for six animals in each group

Group	Plasma (nmol/ml)	Liver (μmol/g tissue)	Kidney (μmol/g tissue)	Heart (μmol/g tissue)
Control	5.5 ± 0.5	0.15 ± 0.02	0.08 ± 0.01	0.07 ± 0.01
Diabetic	7.2 ± 0.6**	0.17 ± 0.03	0.15 ± 0.02***	0.13 ± 0.01***
Insulin treated diabetic	6.5 ± 0.5	0.15 ± 0.02	0.09 ± 0.02	0.08 ± 0.01

***$p < 0.001$, control vs. diabetic.

plasma, 42% in kidney, 28% in heart and 27% in brain. Insulin treated diabetic animals showed no significant change in ascorbic acid and α-tocopherol levels in plasma, liver, kidney, heart and brain when compared to their controls (data not shown).

Conjugated Diene

Significant progressive increases in conjugated diene levels were observed ($p < 0.001$) in the plasma, heart, kidney and brain of diabetic animals, starting from second week, whereas no significant changes in conjugated diene levels were found in the diabetic liver (data not shown). Table IV shows the levels of conjugated diene in the control and diabetic (6 weeks) and insulin treated diabetic animals. Insulin treated diabetic animals showed no significant change in conjugated diene levels in plasma, liver, kidney, heart and brain when compared to their controls.

DISCUSSION

In the present study, we measured the total LMWA antioxidant capacity of various organs during the progression of diabetes. Several investigators have applied this technique to evaluate the total antioxidant capacity of saliva, tissue homogenate after oxidative damage and plasma.[16–20,24,25] We have shown that there is a progressive decrease in total water and lipid soluble LMWA capacity (as measured by the anodic current) in plasma and other organs, with the exception of liver during the progression of diabetes. This is well correlated with the severity of the diabetes and the elevated levels of conjugated dienes. High levels of conjugated dienes have been reported as early indications of oxidative stress.[26] We have shown that there is an increase in the level of conjugated dienes in the plasma, kidney, heart and brain, but not significantly in liver, during the progression of diabetes. Diabetic liver was found to be resistant against oxidative stress. A similar observation in the heart made by Parinandi *et al.*[14] who stated that alterations in fatty acid composition may contribute to the resistance to oxidative stress. The increase in conjugated diene levels after the first week of induction of diabetes in plasma, kidney, brain and heart suggests that increased oxidative stress occurs well after induction of hyperglycemia. The sources of ROS in diabetes are not known, but it is possible that they are derived from autooxidation of glucose and nonenzymatic glycation.[4,5] *In vivo* electron spin resonance spectroscopy studies demonstrated that the diabetic state enhances the generation of free radicals.[27] Young *et al.*[28] reported that increased oxidative stress in STZ-induced diabetes is attenuated by a combination of ascorbate and an iron chelator (desferrioxamine) supplementation. Insulin treatment has been reported to restore to normal the elevated levels of lipid peroxidation end products and the decreased levels of antioxidants, suggesting that the insulin effect may be due to a reduction in the generation of free radicals by glucose autooxidation or from glycated proteins.[6] In the present study, insulin treated diabetic animals showed no significant change in the LMWA capacity and in the

conjugated diene levels in all the organs, clearly demonstrating the hyperglycemia-induced oxidative stress in untreated diabetic animals. Thus, the observed reduction in the overall LMWA capacity in plasma, kidney, heart and brain may be due to increased utilization of LMWA to counteract the hyperglycemia-induced oxidative stress.

Plasma and PBS homogenates of most of the tissues (except heart) showed two anodic waves which represent two classes of reducing equivalents. The first class consists of ascorbate and urate (identified with HPLC-ECD) and the second may include molecules such as histidine, NADH, melatonin, carnosine and L-tryptophan.[18,24] Similarly, lipid extracts of the samples also revealed two anodic waves, indicating two groups of lipid soluble LMWA. We found that β-carotene is a major component of the first anodic wave, while α-tocopherol is a major component of the second anodic wave.[20] In the present study we observed a low level of ascorbic acid in diabetic plasma, liver and kidney which paralleled the decrease in the anodic current. Insulin treated diabetic animals did not show any significant alterations in the ascorbic acid levels. Ascorbic acid has been documented to react directly with aqueous peroxyl radicals or indirectly by reducing α-tocopheryl radical to regenerate α-tocopherol.[29] Hence, the drop in ascorbic acid levels in the diabetic animals could be due to its utilization in order to neutralize hyperglycemia-induced oxidative stress. An unexpected increase in α-tocopherol was found only in the liver of diabetic animals in the second, third and fourth weeks ($p < 0.01$ and $p < 0.001$) after STZ injection. This could account for the fact that no significant change in the level of conjugated dienes was found in diabetic liver. α-Tocopherol, a membrane-bound and naturally occurring antioxidant, has been shown to protect animal tissues against oxidative damage such as lipid peroxidation both *in vivo* and *in vitro*. The association between the lack of oxidative stress displayed by diabetic liver, and an increase in α-tocopherol is not clear. However, Sukalski *et al.*[15] reported a similar increase in α-tocopherol levels in diabetic liver mitochondria, and they also found that diabetic liver mitochondria showed greater resistance to *in vitro* lipid peroxidation. They suggested that the increased α-tocopherol levels associated with diabetes may be due to an alteration in the metabolism or storage of α-tocopherol by the diabetic rats.

In summary, we have demonstrated significant alterations in the overall profile of LMWA capacity and increased conjugated diene levels in plasma, kidney, heart and brain during the progression of diabetes. Insulin treatment restores the altered levels of LMWA and conjugated diene levels thereby suggesting that hyperglycemia induced oxidative stress. The observed resistance to oxidative stress by the diabetic liver may be due to elevated levels of α-tocopherol.

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References

[1] H. Sies (1997) Oxidative stress: oxidant, and antioxidants. *Experimental Physiology*, **82**, 291–295.
[2] J.W. Baynes and S.R. Thorpe (1999) Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes*, **48**, 1–9.
[3] L.W. Oberley (1988) Free radicals and diabetes. *Free Radical Biology Medicine*, **5**, 13–124.
[4] S.P. Wolff and R.T. Dean (1987) Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes. *Biochemical Journal*, **245**, 243–250.
[5] S.P. Wolff, Z.Y. Jiang and J.V. Hunt (1991) Protein glycation and oxidative stress in diabetes mellitus and ageing. *Free Radical Biology Medicine*, **10**, 339–352.
[6] S.A. Wohaieb and D.V. Godin (1987) Alterations in free radical tissue-defense mechanisms in streptozotocin-induced diabetes rat. Effects of insulin treatment *Diabetes*, **36**, 1014–1018.
[7] A.K. Saxena, P. Srivastava, R.K. Kale and N.Z. Baquer (1993) Impaired antioxidant status in diabetic rat liver: effect of vanadate. *Biochemical Pharmacology*, **45**, 539–542.
[8] D.V. Godin, S.A. Wohaieb, M.E. Garnett and A.D. Goumeniouk (1988) Antioxidant enzyme alterations

in experimental and clinical diabetes. *Molecular Cellular Biochemistry*, **84**, 223–231.

[9] L.A. Sechi, A. Ceriello, C.A. Griffin, C. Catena, P. Amstad, M. Schambelan and E. Bartoli (1997) Renal antioxidant enzyme mRNA levels are increased in rats with experimental diabetes mellitus. *Diabetologia*, **40**, 23–29.

[10] R.K. Sundaram, A. Bhaskar, S. Vijayalingam, M. Viswanathan, R. Mohan and K.R. Shanmugasundaram (1996) Antioxidant status and lipid peroxidation in type II diabetes with and without complications. *Clinical Science*, **90**, 255–260.

[11] S.R.J. Maxwell, H. Thomason, D. Sandler, C. Leguen, M.A. Baxter, G.H.G. Thorpe, A.F. Jones and A.H. Barnett (1997) Antioxidant status in patients with uncomplicated insulin dependent and noninsulin dependent diabetes mellitus. *European Journal of Clinical Investigation*, **27**, 484–490.

[12] A. Ceriello, N. Bortolotti, E. Falleti, G. Taboga, L. Tonutti, A. Crescentini, E. Motz, S. Lizzio, A. Russo and E. Bartoli (1997) Total radical trapping antioxidant parameter in NIDDM patients. *Diabetes Care*, **20**, 194–197.

[13] D.H. Mak, S.P. Ip, P.C. Li, M.K. Poon and K.M. Ko (1996) Alterations in tissue glutathione antioxidant system in streptozotocin-induced diabetic rats. *Molecular Cellular Biochemistry*, **162**, 153–158.

[14] N.L. Parinandi, Ed W. Thompson and H.H.O. Schmid (1990) Diabetic heart and kidney exhibit increased resistance to lipid peroxidation. *Biochemistry Biophysics Acta*, **1047**, 263–269.

[15] K.A. Sukalski, K.A. Pinto and J.L. Berntson (1993) Decreased susceptibility of liver mitochondria from diabetic rats to oxidative damage and associated increase in α-tocopherol. *Free Radical Biology Medicine*, **14**, 57–65.

[16] R. Kohen, O. Tirosh and R. Gorodetsky (1992) The biological reductive capacity of tissue is decreased following exposure to oxidative stress: a cyclic voltammetry study of irradiated rats. *Free Radical Research Communications*, **17**, 239–247.

[17] R. Kohen (1993) The use of cyclic voltammetry for the evaluation of oxidative damage in biological samples. *Journal of Pharmacology Toxicological Methods*, **29**, 185–193.

[18] R. Kohen, S. Chevion, R. Schwartz and E.M. Berry (1996) Evaluation of the total low molecular weight antioxidant activity of plasma in health and diseases: a new approach. *Cellular Pharmacology*, **3**, 355–359.

[19] R. Kohen, D. Fanberstein and O. Tirosh (1997) Reducing equivalents in the aging process. *Archives of Gerontology Geriatrics*, **24**, 103–123.

[20] R. Kohen, E. Beit-Yannai, E.M. Berry and O. Tirosh (1999) Overall low molecular weight antioxidant activity of biological fluids and tissues by cyclic voltammetry. *Methods in Enzymology*, **300**, 285–296.

[21] A.A. Like and A.A. Rossini (1976) Streptozotocin-induced pancreatic insulitis; new model of diabetes mellitus. *Science*, **193**, 415–417.

[22] P.A. Motchnik, B. Frei and B.N. Ames (1994) Measurement of antioxidants in human blood plasma. *Methods in Enzymology*, **234**, 269–279.

[23] J.A. Buege and S.D. Aust (1978) Microsomal lipid peroxidation. *Methods Enzymology*, **52**, 302–310.

[24] E. Beit-Yannai, R. Kohen, M. Horowitz, V. Trembovler and E. Shohami (1997) Changes of biological reducing activity in rat brain following closed head injury: a cyclic voltammetry study in normal and heat acclimated rats. *Journal of Cerebral Blood Flow and Metabolism*, **17**, 273–279.

[25] L. Lomnitski, R. Kohen, Y. Chen, E. Shohami, V. Trembovler, T. Vogel and D.M. Michaelson (1997) Reduced levels of antioxidants in brain of apolipo protein E-deficient mice following closed head injury. *Pharmacology Biochemistry and Behavior*, **56**, 669–673.

[26] P.A. Low and K.K. Nickander (1991) Oxygen free radical effects in sciatic nerve in experimental diabetes. *Diabetes*, **40**, 873–877.

[27] T. Sano, F. Umeda, T. Hashimato, H. Nawata and H. Utsumi (1998) Oxidative stress measurement by *in vivo* electron spin resonance spectroscopy in rats with streptozotocin-induced diabetes. *Diabetologia*, **41**, 1335–1360.

[28] I.S. Young, J.J. Torney and E.R. Trimble (1992) The effect of ascorbate supplementation on oxidative stress in the streptozotocin diabetic rat. *Free Radical Biology Medicine*, **13**, 41–46.

[29] J.M. May, Z. Qu and S. Mendiratta (1998) Protection and recycling of α-tocopherol in human erythrocytes by intracellular ascorbic acid. *Archives of Biochemistry and Biophysics*, **349**, 281–289.